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Monitoring F165$_1$ P-Like Fimbria Expression at the Single-Cell Level Reveals a Highly Heterogeneous Phenotype

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F165$_1$ and the pyelonephritis-associated pili (Pap) are two members of the type P family of adhesive factors. They play a key role in establishing disease caused by extraintestinal pathogenic *Escherichia coli* (ExPEC) strains in animals and humans. Both F165$_1$ and Pap are under the control of an epigenetic and reversible switch that defines the number of fimbriated (ON) and afimbriated (OFF) cells within a clonal population. Using the Gfp reporter system, we monitored *in vitro* the level of fluorescence intensity corresponding to the F165$_1$ and Pap fimbrial synthesis. Monitoring individual *Escherichia coli* cells by flow cytometry and by real-time fluorescence microscopy, we identified cells associated with a low or high level of fluorescence intensity and a large amount of cells with partial levels of fluorescence, mostly present in the F165$_1$ system. This mixed population identified through fluorescence intensity could be attributed to the high switching rate previously observed in F165$_1$-positive bacteria. The fimbrial heterogeneous phenotype for these ExPEC could represent increased fitness in unpredictable environments. Our study illustrates that within the large repertoire of fimbrial variants such as the well-characterized Pap, F165$_1$ is an exquisite example of regulatory expression that arms the bacterium with strategies for surviving in more than one particular environment.

Adherence to host cells relies on the synthesis of specialized molecules that play an important role in multiple steps during the infectious process. Adhesins contribute to virulence by promoting the attachment and bacterial colonization on the host cell surface. *Escherichia coli* possesses a wide variety of adhesins, which all differ in structure and in functions. Moreover, a single strain can present more than one adhesin. This implies a complex regulatory network to produce exclusively one adhesin or to simultaneously express more than one.

Phase variation of P fimbriae is a stochastic and reversible switch between an on phenotype and an off phenotype, "all-or-nothing" (ON/OFF), each capable of responding to a specific condition. After cell division, the majority of daughter cells retain the expression phase of the parent; a minority switches to the opposite expression phase. F165$_1$ fimbriae, encoded by the *foo* gene cluster, are members of the type P fimbria family of *Escherichia coli* (1–4). The regulatory region of *foo* shares more than 96% identity with that of the pyelonephritis-associated pili (*pap*) gene. Previous work showed that F165$_1$ fimbriae are essential for virulence and survival during systemic stages of infection in young pigs (5–8). F165$_1$ fimbriae are principally found in infections caused by opportunistic *E. coli* in animals, while Pap fimbriae are found in human clinical cases of urinary tract infections. The phase variation of F165$_1$ fimbriae shares similar features with that of the well-characterized Pap fimbriae (9–11). This includes an ~400-bp intergenic region surrounded by two divergently transcribed genes (*papI* and *papB* for Pap, *fooI* and *fooB* for F165$_1$). It also includes six leucine-responsive regulatory protein (Lrp) binding sites, of which two are GATC sites (referred to as GATC^{prox} and GATC^{dist}, respectively), spaced 102 bp apart. In *pap* and *foo*, phase variation occurs because of methylation-controlled modifications within the regulatory region of the fimbrial operon. This regulation is epigenetic since it does not involve any changes in DNA sequence (12). The differential methylation status of these sequences determines the binding of Lrp. Indeed, the switch from one phenotype to the other arises from the competition between the binding of Lrp at its distal or proximal binding sites and methylation by the Dam methyltransferase at the opposite GATC site (for reviews, see references 9, 10, and 11). When GATC^{dist} is fully methylated, Lrp cooperatively binds to sites 1 to 3, maintaining the cells in the OFF state; when GATC^{prox} is fully methylated, Lrp binds to sites 4 to 6, maintaining the ON state (Fig. 1). Control of *pap* expression also requires the action of PapI, a positive regulator that increases the affinity of Lrp for sites 4 to 6 *in vivo* (11, 13). PapB, the second specific regulator of the *pap* operon, plays an important role by coordinating the expression of the *pBA* and *pI* promoters (11). All together, the combined actions of Lrp, Dam methyltransferase, PapI, and PapB result in a finite probability of each cell to express (ON) or not (OFF) Pap fimbriae just after DNA replication. Sto-

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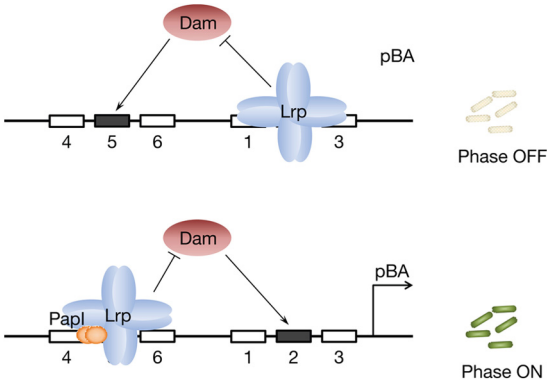

FIG 1 Simplified model of *pap* phase variation. Organization of the regulatory region shared between the *foo* and the *pap* operons, with binding sites for the main regulators depicted according to Hernday et al. (29). [Reprinted from *Molecular Cell*, 12/4, Hernday AD, Braaten BA, Low DA, The mechanism by which DNA adenine methylase and PapI activate the *pap* epigenetic switch, p 947–957, Copyright (2003), with permission from Elsevier.] The Lrp sites are shown as empty rectangles. Gray rectangles represent GATC sites submitted to methylation by Dam, $GATC^{prox}$, and $GATC^{dist}$, which are located within Lrp binding sites 2 and 5, respectively. Localization of Lrp and PapI binding onto their sites and methylation status are also shown for bacteria in either the OFF state or the ON state.

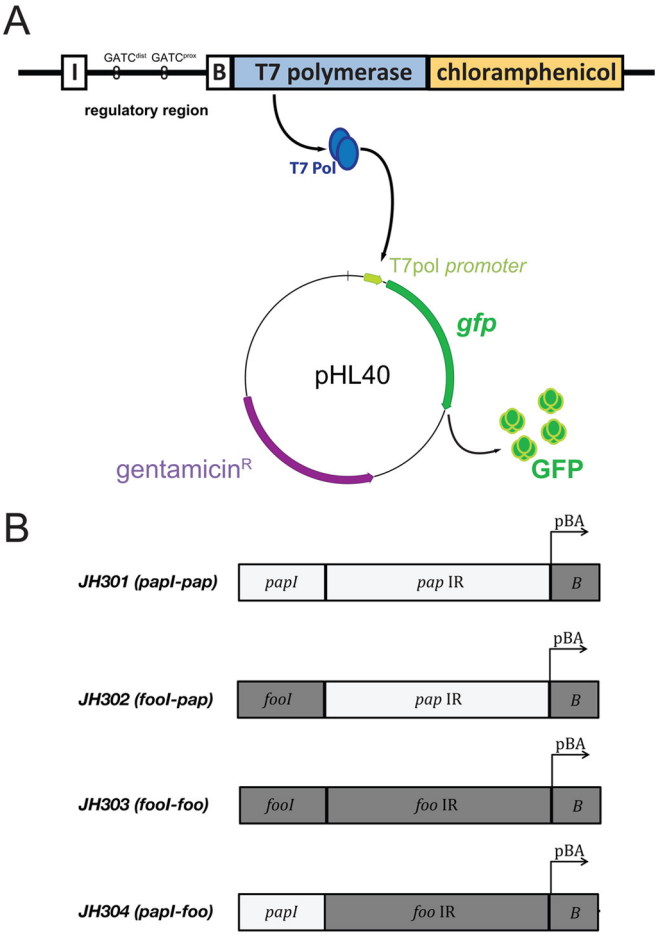

FIG 2 Schematic representation of the reporter system and constructions used in this study. (A) Schematic representation of the reporter system. In order to monitor *foo* and *pap* phase variation, we adapted the genetic amplification system developed by Lim and van Oudenaarden [reprinted by permission from Macmillan Publishers Ltd.: *Nature Genetics* (21), copyright (2007)]. The reporter system was constructed by replacing the chromosomal coding sequence of the fimbrial operon by the sequence of the T7 RNA polymerase (T7 pol). The expression of T7 pol is under the control of phase variation and, in turn, dictates the expression of plasmidic Gfp (under the control of a T7-specific promoter) (21). This system allows an amplified signal and thus improves the signal-to-background ratio. (B) Schematic representation of the regulatory region of strains. *pap* sequences are represented in light gray and *foo* sequences in dark gray.

chastic simulation models have been used for the numerical study of the Pap pilus epigenetic switch to produce predictions regarding the transient switching behavior of Pap switch (14, 15).

In contrast to the *pap* phase variation, where most of the colonies are in the OFF state, the phase variation of *foo* is characterized by a majority of ON colonies (16). We have shown that subtle sequence differences within the promoter proximal regulatory region (when combined with the specific actions of FooI or PapI) cause a significant change in the level of ON cells and that the different expression level of *foo* relative to *pap* relies on the concerted action of Lrp, Dam methylase, FooB, and FooI (17–19). Ultimately, this creates two different phase variation systems: Pap as a slow switcher and $F165_1$ as a fast switcher. However, all these observations have been made by inferring the number of ON and OFF cells from the expression level of colonies (20). To compare $F165_1$ and Pap switching events in single cells, we adapted a genetic amplifier system from Lim and van Oudenaarden (21) to express T7 RNA polymerase (T7 RNAP or T7pol) under the control of the *foo* or *pap* regulatory region. Once T7 RNAP is expressed, it amplifies a T7 RNAP-specific promoter that regulates expression of the green fluorescent protein (*gfp*) gene located on a plasmid (Fig. 2). This system enables single cells to be distinguished according to their fluorescence intensity (low or high) by flow cytometry and fluorescence microscopy. Following cell growth and the level of *gfp* expression over time, we observed distinct patterns of *foo* and *pap* expression that confirmed the difference between switch rates. Moreover, we observed heterogeneous phenotypes in cells expressing T7 RNAP under the control of the *foo* regulatory region. In multiple genealogically related lineages, we also observed by fluorescence microscopy that $F165_1$ ON bacteria after cell division generate cells with a partial expression associated with a high switching rate. The fimbrial heterogeneous phenotype observed for $F165_1$ bacteria could represent increased fitness in unpredictable environments.

MATERIALS AND METHODS

Media and growth conditions. Luria-Bertani (LB) broth and M63 and M9 minimal broths were prepared as described previously (22, 23). When necessary, media were supplemented with antibiotics at the following concentrations (unless otherwise noted): ampicillin (Amp; 100 $\mu g \cdot ml^{-1}$); chloramphenicol (Cam; 10 $\mu g \cdot ml^{-1}$); gentamicin (10 $\mu g \cdot ml^{-1}$); kanamycin (40 $\mu g \cdot ml^{-1}$); tetracycline (Tet; 12.5 $\mu g \cdot ml^{-1}$); and 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-Gal; at a final concentration of 40 $\mu g \cdot ml^{-1}$). For microscopy experiments, M9 minimal salts medium was supplemented with 0.2% glycerol, gentamicin (10 $\mu g \cdot ml^{-1}$), and 2% BD Bacto agar.

Strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. The primers used for strain construction can be found in Table S1 in the supplemental material. To monitor *foo* and *pap* phase variation, we adapted the genetic amplification system developed by Lim and van Oudernaarden (21). The reporter system was constructed by

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
E. coli strains		
NC397	*lacI*::*kan-ter-cat sacB-lacZYA*	53
cHNL135	MC4100 *agn43*::*T7pol*; Cam^r	21
papOFF	MC4100 λ354 lysogen (wild-type *papI-papGCTC*^{p,r,o,x}*-papBA-T7pol*; Cam^r) + pHL40	This study
papON	MC4100 λ354 lysogen (wild-type *papI-papGCTC*^{d,i,s,t}*-papBA-T7pol*; Cam^r) + pHL40	This study
DL4388	MC4100 λ354 lysogen (wild-type *papI-pap-papBA-lacZYA*)	12
JH100	DL4388 *papI′*::*cat-sacB-papBA-lacZYA*	16
JH200	MC4100 λ354 lysogen (*fooI-foo-papBA-lacZYA*)	16
JH201	MC4100 λ354 lysogen (*papI-foo-papBA-lacZYA*)	16
JH204	MC4100 λ354 lysogen (*fooI-pap-papBA-lacZYA*)	16
JH210	MC4100 λ354 lysogen (*fooI-foo-papBA-lacZYA*) + pHL40	This study
JH301	MC4100 λ354 lysogen (*papI-pap-papBA-T7pol*; Cam^r) + pHL40	This study
JH302	MC4100 λ354 lysogen (*fooI-pap-papBA-T7pol*; Cam^r) + pHL40	This study
JH303	MC4100 λ354 lysogen (*fooI-foo-papBA-T7pol*; Cam^r) + pHL40	This study
JH304	MC4100 λ354 lysogen (*papI-foo-papBA-T7pol*; Cam^r) + pHL40	This study
Plasmids		
pKM201	Lambda genes *red* and *gam* under the control of the IPTG[a]-inducible Ptac promoter (Amp^r)	54
pHL32	Reporter plasmid that expresses *gfp* under the control of a T7 RNA polymerase-specific promoter (Kan^r)	21
pHL40	pHL32 derivative that possesses the gentamicin resistance cassette	This study

[a] IPTG, isopropyl-β-D-thiogalactopyranoside.

replacing the chromosomal coding sequence of the fimbrial operon by the sequence of the T7 RNA polymerase (T7pol). The expression of T7 pol is under the control of phase variation and, in turn, dictates the expression of plasmidic *gfp* mut3.1 (under the control of a T7-specific promoter) (21). Briefly, the T7 polymerase coding sequence replaced *lacZ* under the control of *foo* and *pap* fimbrial promoters of strains that were obtained through lambda *lacZ* fusion vector lysogeny, JH200 (*fooI-foo-lacZ*), JH201 (*papI-foo-lacZ* mutant), DL4388 (*papI-pap lacZ*), and JH204 (*fooI-pap lacZ* mutant) (12, 16), and plasmid pHL40 carrying *gfp* and resistance to gentamicin was inserted in these constructs. This resulted in strains JH301 (*papI-pap-T7pol*) and JH303 (*fooI-foo-T7pol*) and *fooI-papI* swapping strains JH302 (*fooI-pap-T7pol*) and JH304 (*papI-foo-T7pol*) (Table 1 and Fig. 2). Control mutant strains locked OFF (papOFF) and locked ON (papON) were generated using the same procedure, with the insertion of a 459-bp DNA fragment, which contains a GCTC point mutation in the GATC site of the proximal or distal of the *pap* intergenic region, respectively (12) (Fig. 2).

Briefly, for the construction of the last set of strains, a PCR fragment corresponding to the T7 RNA polymerase gene (*T7pol*) and the chloramphenicol resistance gene (Cam^r gene) was first amplified from strain cHNL135 using lacZ-T7pol_fwd and lacZ-CAM_R. The PCR product was then recombined into strains JH200 (*fooI-foo*), JH201 (*papI-foo* mutant), DL4388 (*papI-pap*), and JH204 (*fooI-pap* mutant) using the λ red recombination system (12, 16, 24). Recombinant strains were selected for resistance on LB-Cam agar and then transformed with *gfp* reporter plasmid pHL40. Plasmid pHL40 was constructed by replacing the kanamycin resistance cassette from plasmid pHL32 (21) by a gentamicin resistance cassette. Briefly, a gentamicin resistance cassette was first amplified from plasmid pUCGM using primers SacI-genta-F and AatII-genta-R. Plasmid pHL32 was then digested using the endonucleases AatII and SacI (New England BioLabs), and after purification, the DNA fragment containing the T7 RNA polymerase-specific promoter and *gfp* was ligated with the PCR-amplified gentamicin resistance cassette to generate plasmid pHL40. For cloning procedures, PCR amplification was carried out using the FideliTaq PCR MasterMix (GE Healthcare).

Flow cytometry acquisition and analysis. Cells were streaked onto LB plates with appropriate antibiotics. After 24 h of growth at 37°C, a single colony was washed in M9 containing 0.2% glycerol and centrifuged at 8,000 rpm for 5 min. The pellet was then used to inoculate an overnight culture in the same medium. After another 24 h of growth, cells were washed and transferred into 1 ml sterile phosphate-buffered saline (PBS), and ON and OFF single cells were separated using a Becton Dickinson FACSAria flow cytometer (488-nm excitation laser; 525-nm emission filter). ON and OFF populations (from 10,000 to 100,000 individual cells) were then resuspended in 1 ml of fresh M9 medium containing 0.2% glycerol and allowed to grow at 37°C for 20 h. These populations were then analyzed by flow cytometry. Bacterial populations were tracked using a logarithmic setting adjusted for bacterial size (forward scatter/side scatter [FSC/SSC]) and by fluorescence with a laser emitting at 488 nm (FL1-H) and collected at wavelengths of 530/30 nm (25).

The gate for the detection of fluorescence signals was set such that cells under investigation were considered positive when their fluorescence intensity (FL-1 height) exceeded that of all but a very small fraction of the negative-control population of nonfluorescent strain JH210 (*fooI-foo-lacZYA*) lacking T7pol but having plasmid pHL40. The gate of the positive-control population of fluorescent strain papON, constitutively expressing T7pol and having plasmid pHL40, was used to set up the instrument. Strains papOFF and papON were considered our biological control since they are blocked in two opposite phases (16). Data from 10,000 to 100,000 individual cells were collected for each sample and analyzed with a Becton Dickinson FACSCanto II flow cytometer (488-nm excitation laser; 525-nm emission filter). FACSCanto data were converted to ASCII format using mean fluorescence intensity (MFI; E. Martz, University of Massachusetts, Amherst) and analyzed with FlowJo software (Tree Star, Inc.).

Real-time microscopy. (i) Microscopy sample preparation. Bacteria were first cultivated on LB agar and then isolated on minimal M9 medium with glycerol and grown for 24 h at 37°C. Colonies were harvested and resuspended in 1 ml sterile PBS solution. The prewarmed cell suspension was then diluted in PBS at 1/100. Five microliters of the bacterial suspension was dropped onto the agar strip.

An adhesive silicone isolator (Grace Bio-labs) (19 mm by 32 mm by 1 mm) was affixed onto a microscope slide in order to create a chamber that contained the solid medium. A volume of 700 μl culture medium consisting of M9 glycerol medium with 2% agar was poured into the silicone isolator. A coverslip (22 mm by 60 mm by 60 mm; Fisher) coated with Sigmacote (Sigma) was then placed over the isolator. Once the agar was solidified, the coverslip was removed. The now-flat-surfaced agar was trimmed into a thin strip about 19 mm wide and 15 mm long.

(ii) Image acquisition. Microscope slides and bacterial suspensions were placed in the 37°C incubation chamber of the Zeiss Axiovert inverted

microscope, so as to limit the cold shock when the cells would be transferred onto the microscope slides. A 5-μl volume of the bacterial suspension was dropped onto the agar strip. The sample was immediately closed using another coverslip and was ready for observation.

The observations followed the growth of a single ON phase cell. The growth was followed for 10 h at 37°C, with pictures taken every 10 min; both differential interference contrast (DIC) and fluorescence images were captured.

(iii) Data analysis. Images were reviewed and analyzed using the MATLAB analysis package Schnitzcells, kindly provided by M. Elowitz (California Institute of Technology), that segments and tracks cells in a frame-to-frame method (26). This process yields quantitative expression data on cell lineages, which can illustrate dynamic expression profiles.

RESULTS

Single-cell measurement of *foo* and *pap* phase variation reveals a heterogeneous population. To detect *foo* and *pap* switching events in single cells, we used the genetic amplification system developed by Lim and van Oudenaarden (21). The expression of T7 RNAP is under the control of phase variation and, in turn, dictates the expression of a plasmidic *gfp* (under the control of a T7-specific promoter) (Fig. 2) (21). This system facilitates single-cell measurements of transcription from weak bacterial promoters in their native positions. The levels of fluorescence intensities were distinguished by flow cytometry for the following strains: JH301 (*papI-pap-T7pol*); JH303 (*fooI-foo-T7pol*); *fooI-papI* swapping strains JH302 (*fooI-pap-T7pol*) and JH304 (*papI-foo-T7pol*), carrying the plasmid pHL40 (carrying *gfp* under a T7 pol-specific promoter) (Fig. 2; Table 1).

The fluorescence of the bacterial populations was analyzed by flow cytometry. The bacteria were also sorted according to their fluorescence intensity (low and high fluorescence). The state of populations after growth of these sorted progenitor bacteria was analyzed by flow cytometry (Fig. 3). Negative-control JH210 and papOFF and positive-control papON strains were used as controls (Table 1) (16). The histograms of single-cell fluorescence measured by flow cytometry showed different levels in *gfp* expression between strains (Fig. 3). As expected, the negative-control strain papOFF showed almost no fluorescent cells (Fig. 3B) and the positive-control strain papON consistently showed a high level of fluorescence (10^4 to 10^5 arbitrary units [AU]) (Fig. 3B). In contrast, constructs JH301 (*papI-pap*) and JH303 (*fooI-foo*) presented different levels of fluorescent intensities (Fig. 3C and D). These were defined as low (ranging from 0 to 10^3 AU), partial (ranging from 10^3 to 10^4 AU), or high (ranging from 10^4 to 10^5 AU) fluorescence intensity.

After growth of sorted bacteria with high-level fluorescence from strain JH303 (*fooI-foo*), a population showing partial to high levels of fluorescence (~10^4 AU) (Fig. 3M) was detected, while after growth of sorted bacteria with a low level of fluorescence, JH303 exhibited two populations, constituted by a minor proportion of bacteria with a low level of fluorescence and a major proportion of bacteria with a partial level of fluorescence (Fig. 3I). Construct JH301 (*papI-pap*) produced mainly a population with a low level of fluorescence regardless of the starting sorted population (Fig. 3G and K). These results correlate with previous observations that showed a higher proportion of ON colonies for *foo-lacZ* than for *pap-lacZ* (16, 17, 27). Like construct JH303 (*fooI-foo*), swapping strain JH304 (*papI-foo*) exhibited two clearly marked fluorescence peaks (Fig. 3F). However, the two populations differed in the fluorescence intensity (partial level for strain JH303 and high level for strain JH304). When starting from sorted bacteria with low fluorescence, strain JH303 showed a majority of cells with partial fluorescent intensity while strain JH304 showed a majority of bacteria with a low level of fluorescence) (Fig. 3I and J). The swapping strain JH302 (*fooI-pap*) showed a fluorescence tendency profile similar to that of JH303 (*fooI-foo*), with an important subpopulation of cells having partial-to-high fluorescence levels (10^3 to 10^4 AU) (Fig. 3H and L), regardless of the starting phenotype.

These observations made at the cellular level confirmed previous observations that showed a higher proportion of ON colonies for *foo-lacZ* than for *pap-lacZ* strains (16). Moreover, flow cytometry data indicate that *foo* individual cells derived from an initial low level of fluorescence present two distinct populations, one with low fluorescence intensity and the second with partial intensity. This specific population suggests that the light blue colonies observed when the *lacZ* reporter system is under the control of the *foo* regulatory region result in a mixed population of white (OFF) and dark blue (ON) individual cells (16, 27, 28).

The FooI/PapI regulators influenced the phenotype of the swapping strains compared to their respective parental strains. Indeed, the swapping construct *fooI-pap* (JH302) had a more important subpopulation of cells with partial to high levels of fluorescence, with greater similarity to that of the *fooI-foo* strain than to the parental *papI-pap* strain. Moreover, the *papI-foo* swapping strain (JH304) exhibited a striking difference in the maintenance of low fluorescence intensity from its parental *fooI-foo* strain (Fig. 3I and J).

Thus, the expression of *gfp* seemed to be inherited in an epigenetic manner, similar to the measurement of phase variation using a fimbrial promoter *lacZ* fusion in colonies (16, 17, 29). As reported here, the Pap construct presented a majority of cells with low levels of fluorescence that can be assimilated to an OFF phase. Reciprocally, the presence of an important intermediate fluorescence subpopulation in $F165_1$ indicates that an intermediate expression phase occurs during the switch. The influence of PapI homologues specifically on the switch rate and the proportion of the intermediate phase cells is revealed in the swapping strains.

Real-time analysis of *pap* and *foo* phase variation. To characterize the distribution of the *foo* and *pap* operon expression level at a single time point, individual *E. coli* cells were observed by real-time microscopy. Phase variation was monitored as the cells grew and divided, because type P fimbria phase transition requires DNA replication (11, 29–31) (see Fig. S2 in the supplemental material). Cell growth and the level of green fluorescent protein (GFP) expression were monitored over time, and the genealogical relationships were determined within the population using image analysis. JH301 (*papI-pap*), JH302 (*fooI-pap*), and JH303 (*fooI-foo*) growing in an M9 glycerol medium were spread onto agar and then imaged using phase-contrast and fluorescence microscopy. All strains demonstrated regular growth under the microscope. The experiments were started with cells presenting an initial high fluorescent intensity. Microcolonies of 87 to 181 cells were formed over a period of 10 h with a generation time of 80 to 93 min during the exponential phase.

We observed distinct patterns of *foo* and *pap* expression in multiple genealogically related lineages. Similar to results observed by flow cytometry, distinctive patterns of fluorescence were observed: a pattern with low mean fluorescence (0 to 2 AU) and a pattern with a high level of fluorescence (≥4 AU), corresponding

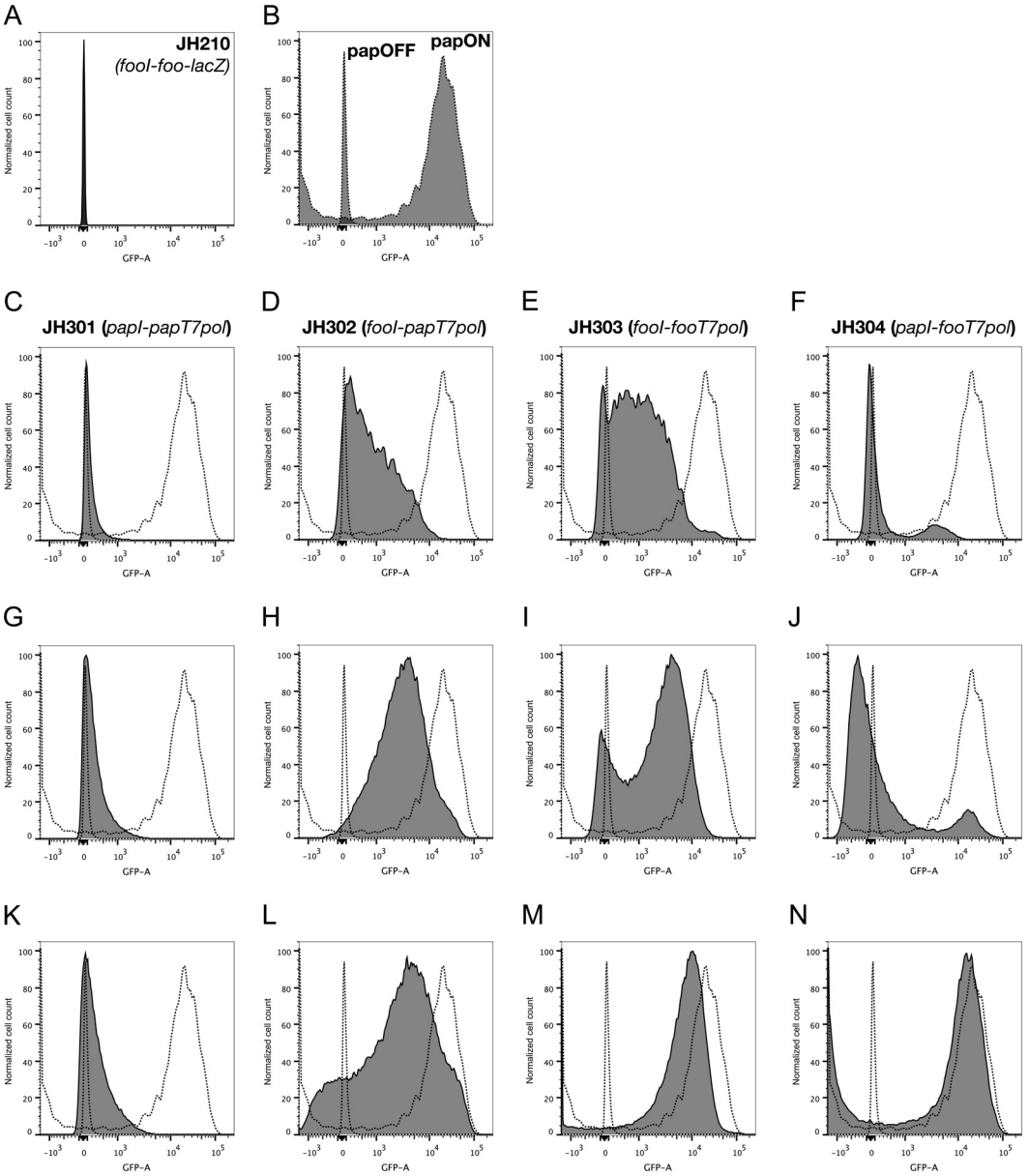

FIG 3 Histograms of single-cell fluorescence for *foo* and *pap* strains correspond to OFF, partial, and ON expression states. A colony isolated from a rich medium plate is streaked onto M9 glycerol minimal medium. After 24 h of incubation at 37°C, colonies are resuspended in PBS and bacteria corresponding to the ON and OFF populations are sorted, using a flow cytometer, and reinoculated to liquid M9 minimal medium. These are the progenitor cells from which the populations are analyzed by flow cytometry after incubation. Top panels correspond to controls: control OFF strain JH210 (*fooI-foolacZ*) (A) and control OFF strain papOFF and ON strain papON containing the pHL40 reporter plasmid (B). Shown are histograms for strain JH301 (*papI-pap*) (C, G, and K); swapping strain JH302 (*fooI-pap*) (D, H, L); strain JH303 (*fooI-foo*) (E, I, M); and swapping strain JH304 (*papI-foo*) (F, J, N). Three populations with different fluorescence intensity levels corresponding to low intensity (OFF state), partial intensity (intermediate state), and high intensity (ON state) can be observed. (C to F) Profile histograms for strains JH301 to JH304 before separation; (G to J) profile histograms for strains JH301 to JH304 after separation (derived from the negative population); (K to N) profile histograms for strains JH301 to JH304 after separation (derived from the positive population). Histograms presented with dashed lines indicate the positions of the two control strains papOFF and papON.

to the OFF and ON states, respectively, and another pattern with partial levels of fluorescence (ranging from 2 to 4 AU) (Fig. 4). Three different subpopulations were observed in different lineages of JH303 (*fooI-foo*) (see Movie S3 in the supplemental material). Over time, when different lineages were being monitored, many daughter cells showed partial fluorescence levels and only a subset of daughter cells presented a clear ON or OFF fluorescence phenotype. While lineages from several highly fluorescent progenitors of JH301 (*papI-pap*) rapidly switched to low fluorescent levels upon division, most of their daughter cells remained at basal fluorescence intensities (see Movie S1 in the supplemental material). Only a small subset of cells switched back from low to high fluorescence levels. The swapping strain JH302 (*fooI-pap*) (see Movie S2 in the supplemental material) behaved in a manner similar to that of JH303 (*fooI-foo*), whereby the majority of the daughter cells maintained intermediate levels of fluorescence.

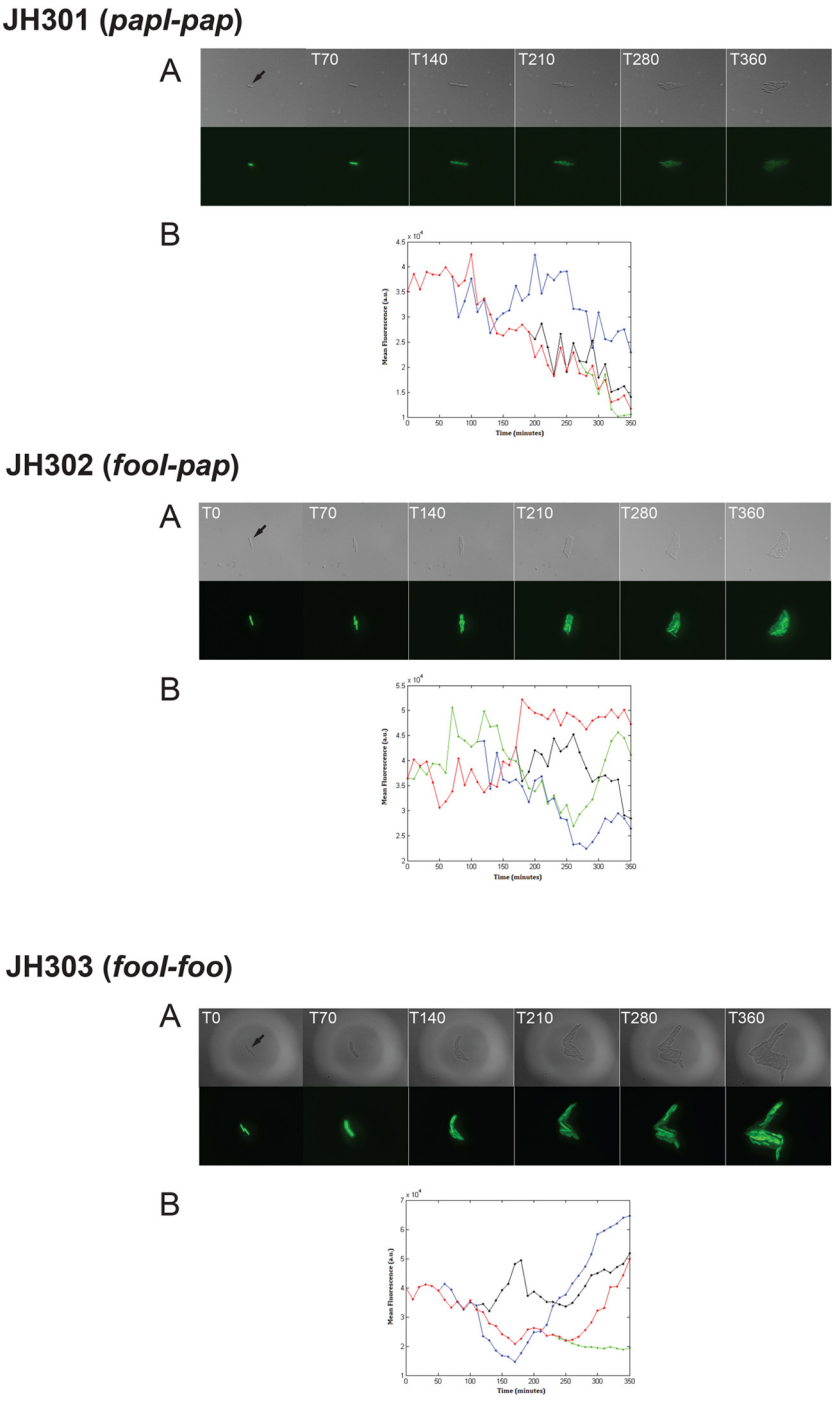

FIG 4 ON switching in a growing microcolony. Top panel, JH301 (*papI-pap*), middle panel, JH302 (*fooI-pap*), bottom panel JH303 (*fooI-foo*). (A) Differential interference contrast (DIC) and fluorescence images of JH301 (*papI-pap*), JH302 (*fooI-pap*), and JH303 (*fooI-foo*), starting from an ON cell (black arrow). A fluorescent *foo* cell develops into an intermediate-fluorescence subcolony, indicating frequent switching events, while a fluorescent *pap* cell develops into a low-fluorescence subcolony, indicating mostly an ON-to-OFF switch event. (B) GFP expression history of the four lineages as represented by different-colored lines. Starting from an ON cell, most of the JH303 (*fooI-foo*) and JH302 (*fooI-pap*) lineages show an increase or variable intensity fluorescence as the cells divide, whereas JH301 (*papI-pap*) lineages rather show a monotonic decrease of fluorescence. The *y* axis represents the mean of fluorescence (in arbitrary units [a.u.]), and the *x* axis represents the time (in minutes). In each panel, the red line represents the mother lineage.

Quantitative expression data on cell lineages using the Schnitzcells program (26) illustrated the dynamic expression profiles of *foo* and *pap*. The lineage of the cells and their Gfp expression level revealed different intensity fluorescence patterns (Fig. 5). JH302 (*fooI-pap*) and JH303 (*fooI-foo*) showed populations with a heterogeneous level of expression of fluorescence, while JH301 (*papI-pap*) showed a marked preference for the OFF state and a monotonic decrease in fluorescence expression within the whole

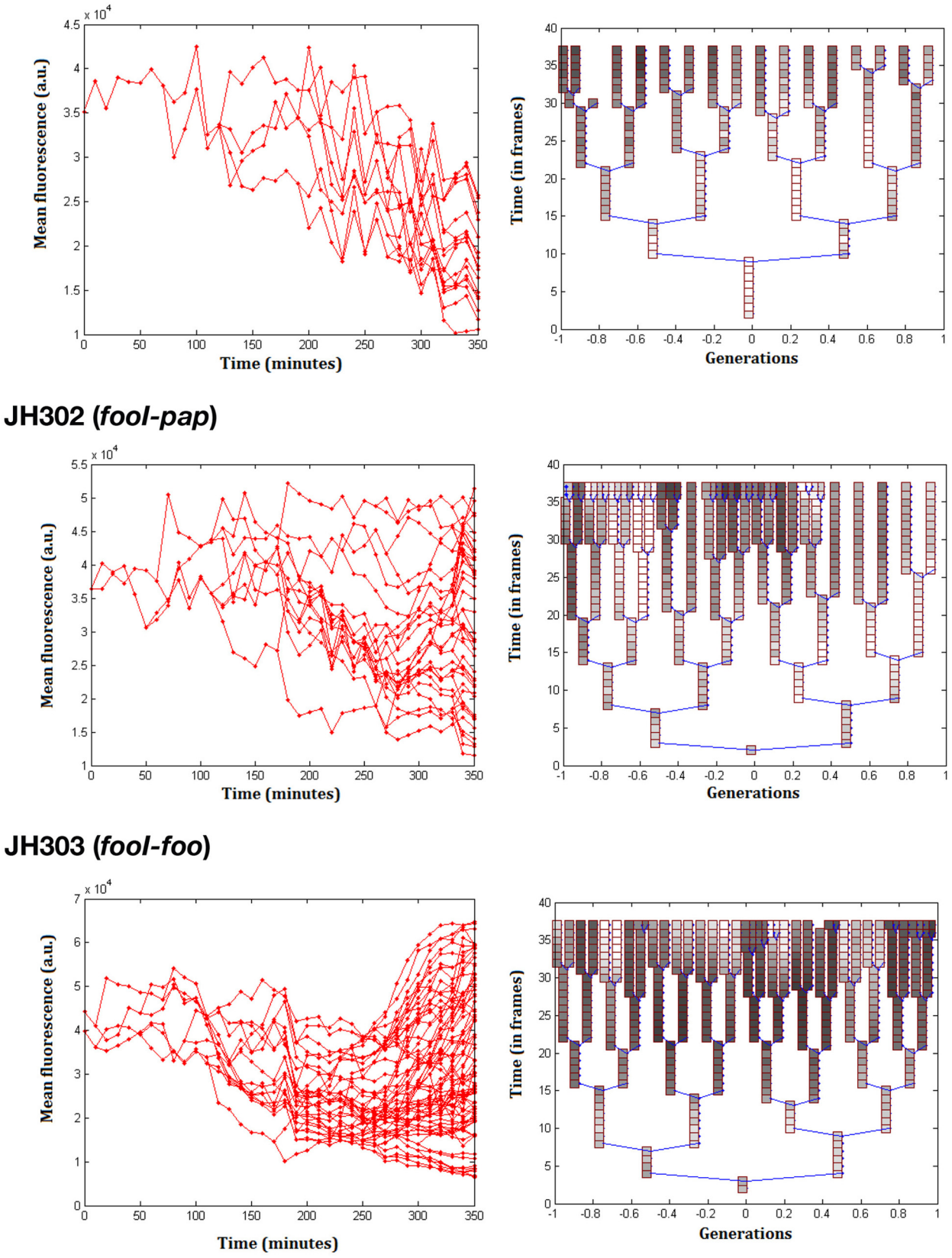

FIG 5 Overall population expression level from the growth of microcolonies. (Left) Overall expression of fluorescence over the course of the experiment for each strain. At each division as the cells divide, JH303 (*fooI-foo*) and JH302 (*fooI-pap*) lineages show an increased or variable intensity in fluorescence whereas JH301 (*papI-pap*) lineages rather show a monotonic decrease of fluorescence. (Right) Lineage trees depicting generations of strains JH301 (*papI-pap*), JH302 (*fooI-pap*), and JH303 (*fooI-foo*). The length of the bars represents division time with each square a picture frame. Each graph starts at the bottom, with cells going from white (ON) to black (OFF).

progeny. In the case of JH301 (*papI-pap*), daughter cells switching from ON to OFF phase inherited GFP protein, T7pol, and mRNA, as observed for type 1 phase variation (32). They were then diluted during subsequent divisions of OFF cells, which explains the monotonic decrease of fluorescence. The heterogeneous fluorescence observed in JH302 (*fooI-pap*) and JH303 (*fooI-foo*) lineages reflected the higher switch rates. Within lineages of these strains, the daughter cells often switched after division to the other phase. These cells can express variable levels of GFP depending on the prior state of the progenitor.

DISCUSSION

Single-cell measurement of *foo* and *pap* expression reveals a higher complexity in phase variation. Using flow cytometry and real-time microscopy, we confirm the observation that *foo* expression ($F165_1$ fimbriae) gives rise to a higher level of ON cells than does expression of *pap* (Pap), but the observation of *foo* phase variation emphasizes the existence of a more heterogeneous population. After OFF cell division, daughter cells can retain the expression phase of the parent cells or switch phase to give rise to an intermediate phase. The different patterns of fimbrial expression could be explained by a derived model proposed by Adiciptaningrum et al. (32). In this model, a single switching event combines chromosomal replication with the epigenetic inheritance of expressed fimbrial protein, mRNA, and their dilution by growth of OFF cells (11). In addition, we propose that the frequency of switching plays a critical role in the significance of the intermediate phase. In that way, *foo* cells demonstrate a high switch rate with variable levels of *foo* expression depending on the prior state of the progenitor. Moreover, the partial expression of *gfp* is linked to a high switching frequency during replication in a cell lineage (32) (Fig. 5).

While a prominent low fluorescence level (OFF phase) characterizes the *pap* construct (16), the *foo* construct gives rise to a wider distribution of expression, not only with low and high fluorescence levels (OFF and ON phases, respectively), but also with more cells expressing partial-to-high levels of fluorescence. Thus, the heterogeneous phenotype observed mainly with *foo* could be attributed to frequent phase variation events correlated to bacterial division events. Starting from an ON cell, the daughter cell that switches to the OFF state inherits only GFP protein and mRNA. This can result in an intermediate expression state. Thus, subsequent division of OFF cells can decrease the baggage of GFP and mRNA. As observed for *pap*, characterized by less frequent switching events and cells in the OFF state, it results in a monotonic decrease of fluorescence. In the case of the *foo* construct, where frequent switching events occur during cell divisions, the lineages express a partial fluorescence baseline. The inheritance of molecules for the progenitor cells was suggested in type 1 fimbriae, in which distinctive patterns of expression in genealogically related lineages were observed during phase switching by DNA inversion (10).

Phase variation measured frequencies in *E. coli* K-12 bearing the *pap* operon, however, were reported not to be predictive of the fimbriation levels for several uropathogenic *E. coli* isolates (33). Indeed, phase transition frequencies for the two *pap* clusters of uropathogenic *E. coli* CFT073 were shown to be different from those of *pap* clusters cloned in K-12 (33). Our previous studies on phase variation of $F165_1$ in clinical strains showed that a majority of cells were in the ON phase (18). Similar to *foo* recombinant strains, we observed that $F165_1$-producing clinical animal strains are producing fimbriae at different levels, although the proportion of OFF cells was higher than that in *E. coli* K-12 (see Fig. S3 in the supplemental material). Different regulatory networks controlling *papI* expression in clinical isolates and the expression level of PapI homologue could influence phase variation transition rates (33).

Studies on *pap* phase variation showed that the competitive Lrp and Dam binding to the regulatory region dictates the stability of Lrp to the proximal region and that this competitive binding is important for the heritability of the OFF state (34). We previously showed that the high level of ON cells found during $F165_1$ phase variation is due to the lack of a stable interaction between Lrp and the *foo* proximal repressor region (16). This region contains subtle differences in sequence compared to that of *pap*. These differences in combination with the local regulator FooI promote the transit of Lrp toward its activator distal region after each cell cycle (16).

During *pap* and *foo* fimbrial phase variations, the competition between Dam and Lrp for sites at each replication event represents an opportunity for Lrp to switch from the activator region to the repressor region (see Fig. S2 in the supplemental material) (23). Lrp dissociation from DNA allows Dam to gain access and to methylate the GATC sites residing in the activator and repressor regions. Therefore, each replication event represents a possibility of a switching event (29). The frequent switching in *foo* is due to a weak affinity of Lrp for its *foo* proximal region that facilitates the OFF-to-ON switch, whereas the formation of a particularly stable complex between Lrp and the *pap* proximal region maintains the OFF state through generations (16) (Fig. 5). Differential methylation assays (28) also correlated the switch rate with the population methylation status of the GATC sites of the fimbrial regulatory region (see Fig. S2 to S4 in the supplemental material). The fraction of hemimethylated sites of both repressor and activator regions is more important in *foo* than in *pap*, suggesting a frequent change of the methylation DNA status during *foo* cell division (see Fig. S2 to S4 in the supplemental material). In contrast, the $GATC^{prox}$ site of the repressor region of *pap* remains methylated in greater proportion than the $GATC^{dist}$ site of the activator region. This correlates with the prevalent OFF state of *pap* cells (Fig. 6).

Multistep epigenetic switches with biased transition rates may exist in various configurations, with an ON-partial-OFF level of expression related to the state of methylation and protein binding to specific sites, as previously demonstrated with *agn43* (21). Lim and van Oudenaarden (21) showed that the switching mechanism of *agn43* requires the presence of several intermediate states that separate the ON and OFF states. The process of *agn43* switching shares some similarities with the *pap*-like fimbrial system, such as its dependence on the methylation state of GATC sequences determined by competitive binding between Dam and the repressor OxyR. The partial states occur during the hemimethylated state and the intermediate unmethylated state. The unmethylated switch region can be remethylated to promote the ON phase or bound by the OxyR repressor to result in the OFF expression (21).

FooI induces a higher switching rate than PapI. The specific regulators FooI and PapI are essential for phase variation (27, 29, 35), and they also influence the phase variation rate (16). The swapping of PapI by FooI in the *pap* background changed the expression to that of *fooI-foo* (Fig. 3 to 5). Reciprocally, the swapping of FooI by PapI in the *foo* background changed the expression

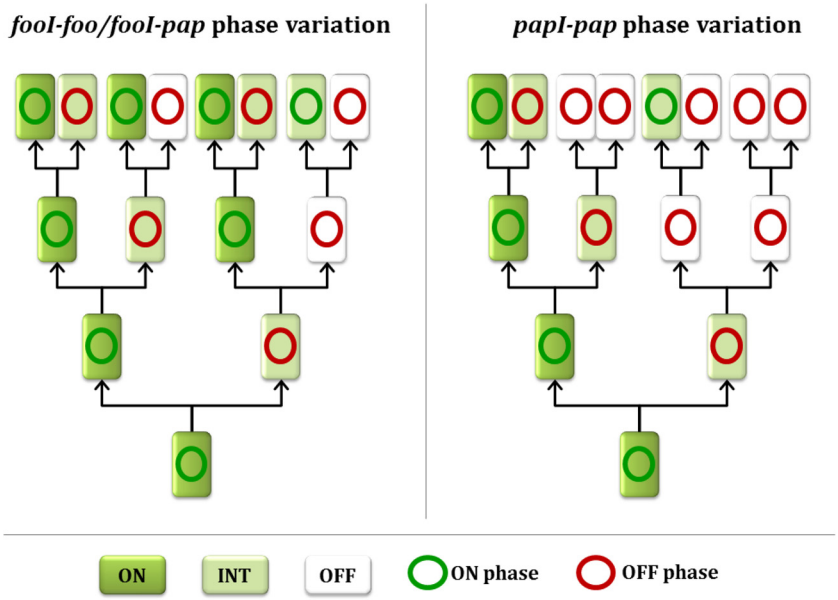

FIG 6 Fimbrial expression and DNA replication: model for *foo* and *pap* switching event during division, including the intermediate phase. $F165_1$ and Pap are both under the control of an epigenetic and reversible switch that defines the number of ON and OFF cells within a clonal population. Slow-growing *E. coli* cells inheriting a single chromosome (replicated bidirectionally) can be submitted to phase switching after DNA replication. Starting from an ON cell, the daughter cell that switches to the OFF state inherits only GFP protein and mRNA. This results in an intermediate expression state. The baggage of GFP and mRNA can be diluted by subsequent division of OFF cells and thus decreases. Characterized by less frequent switching event and cells in the OFF state, the *pap* phenotype results in a monotonic decrease of fluorescence. With frequent switching events during cell divisions, the lineages of *foo* cells express a partial fluorescence baseline. Thus, three phenotypes appear, i.e., ON, OFF, and intermediate (INT). Green circles represent the ON state and red circles the OFF state of the operon. Gradation of green inside cells represents different Gfp expression levels (dark green, high level; light green, intermediate level), while white cells are cells with no Gfp expression.

to that of *papI-pap* (Fig. 3I and J). PapI is a nonspecific DNA-binding protein (16, 36, 37), and it can directly interact with Lrp. We and others have previously shown that a low concentration of FooI and PapI significantly increases the binding and stability of Lrp at both sites in the repressor and activator regions (16, 29, 35). The Lrp-PapI complex is sufficient for the methylation-dependent affinity change to occur (38). Since Lrp alone can form a stable complex with the *pap* repressor region, the action of PapI is more sensitive at the *pap* activator region for maintaining the cells in the ON state. In contrast, the action of FooI upon Lrp is similar in the *foo* binding sites of both regions. This results in a high *foo* switch rate of phase variation. The action of FooI might also influence Lrp binding to *pap* regulatory region concurring with a higher switch rate than with PapI. We previously showed that the high level of ON cells found during $F165_1$ phase variation is due to altered stability of the DNA complex formed by Lrp at its repressor binding sites 1 to 3 and that the nature of the PapI homologue influences the phase variation event (16).

Interestingly, PapI and FooI differ by one single amino acid at position 17 (change of D to N) (21, 29, 30). Totsika et al. (39) showed that variants similar to FooI and PapI have different influence patterns on cross-activation of fimbrial operons. PapI and FooI are members of the Fae-like protein family (PF04703), which includes orthologs of PapI, which are winged helix-turn-helix (wHtH) proteins (37). The third helix has been identified as the one for DNA recognition (37), while the other two serve to provide additional contact. The amino acid difference residing before the second helix might influence the structure of PapI-like proteins and their interaction with Lrp (37, 40). This could account for differences in DNA interaction of PapI-Lrp complexes, which were reported to have specificity for DNA sequence (16, 39, 41). As proposed by Totsika et al., sequence changes in all three interacting factors (PapI-like protein, Lrp, and DNA regulatory sequence) have an impact on their ability to activate fimbrial gene expression (42; this study), without precluding the possibility that other factor(s), required for fimbrial phase switching, might also play a role.

The host as a changing environment. Bacteria experience frequent changes of environment during the infection cycle. One means to rapidly adapt is stochastic switching: a bacterial lineage will stochastically produce a variety of genotypes, so that some descendants will survive if the environment changes. By investigating the behavior of single cells, this study has shown that the stochastic system of phase variation at an individual level has some influence at the population level. We provide evidence that $F165_1$ bacteria could be in ON, OFF, and also partial phases. It has been proposed that population heterogeneity increases the fitness in unpredictable environments (43, 44). Being able to keep a set of *foo* cells in an intermediate expression state would allow the population as a whole to adapt quickly to changing environments. This may work as a kind of bet hedging (45, 46), allowing a given genotype to express multiple phenotypes. One phenotype may be better adapted to the current environment, while others are prepared for future environmental changes under which they may gain higher fitness (47).

In large populations, switching between two phenotypes creates a bimodal population, even though one phenotype might be more fit than the other in the current environment. However, the

unfit subpopulation enables the entire population to hedge its bets in case of future environmental change (48). It is therefore possible that some *E. coli* strains have increased fimbrial switch rates to circumvent host environmental changes.

Acar et al. showed that fast-switching populations outgrow slow switchers when the environment fluctuates rapidly, whereas slow-switching phenotypes outgrow fast switchers when the environment changes rarely (49). In this regard, virulent uropathogenic *E. coli* strains, found at specific sites of infection, may require a stringent control of Pap fimbria production. Similarly, the rapid fimbrial switching of $F165_1$-ExPEC opportunistic strains may enhance their adaptation to fluctuating environments and contribute to their fitness.

Thus, the adaptation to fluctuating environments could compensate the limited arsenal of virulence factors, and the partial state of *foo* expression might give a fitness advantage under some conditions (50).

Using a mathematical model, Gaal et al. showed that asymmetry in the selection pressure between the environments selects against the evolution of stochastic interphenotype switching if the selection pressure is small in at least one of the environments (51). Moreover, they showed that the parameter range for which homogeneity is the favored strategy depends not only on the strength and asymmetry of selection but also on the duration and asymmetry of environmental durations.

Pilus expression of *E. coli* is both beneficial and detrimental. Pili enable *E. coli* to bind to host epithelial cells, establish colonies, and feed off host organisms. Without the binding capabilities of pili, *E. coli* colonies would be more easily flushed from the host. On the other hand, pili may trigger the host immune response. To what extent generating such a heterogenous bacterial population is beneficial for an opportunistic organism remains unknown. Munsky et al. suggested that switching phenotypes could be beneficial for any population descending from a single ancestor to have different pilus expression phenotypes (52). In this regard, urinary tract infection strains producing Pap fimbriae contain an arsenal of virulence factors, including many fimbrial determinants. To conclude, comparing *foo* and *pap* phase variation is another example of how different rates of stochastic switching between phenotypes confer different long-term fitness levels in any particular fluctuating environment.

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